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(54) Title: METHODS AND AGENTS FOR DETECTING THE PROBABILITY OF THE FUTURE OCCURRENCE OR PROGRESSION OF DISEASES THAT ARE ASSOCIATED WITH A DISORDER OF THE NO METABOLISM

(54) Bezeichnung: VERFAHREN UND MITTEL ZUM NACHWEIS EINER WAHRSCHEINLICHKEIT DES ZUKÜNFTIGEN AUFTRETENS ODER FORTSCHREITENS VON ERKRANKUNGEN, DIE MIT EINER STÖRUNG DES NO-STOFFWECHSELS EINHERGEHEN

(57) Abstract: The invention relates to methods and agents for detecting the probability of the future occurrence or progression of diseases that are associated with a disorder of the NO metabolism.

(57) Zusammenfassung: Verfahren und Mittel zum Nachweis einer Wahrscheinlichkeit des zukünftigen Auftretens oder Fortschreitens von Erkrankungen, die mit einer Störung des NO-Stoffwechsels einhergehen.

Description

"Methods and agents for detecting the probability of the future occurrence or progression of diseases that are associated with a disorder of the NO metabolism."

The invention relates to methods for detecting the probability of the future occurrence or progression of diseases that are associated with a disorder of the NO metabolism. The invention furthermore relates to agents for detecting endogenous methyl arginines in biological fluids.

The endogenous methyl arginines ADMA and SDMA are derivatives of the amino acid L-arginine. L-Arginine is the precursor for the formation of nitric oxide (NO) in the human body. NO in turn is an important physiological mediator in the cardiovascular system and other organ systems, which is involved in the regulation of blood pressure and vascular resistance, adhesion and aggregation of platelets, adhesion of leucocytes and monocytes and the proliferation of vessel smooth muscle cells (Böger et al., Atherosclerosis 1996; 127: 1-11). NO also plays an important physiological part in erection. In cardiovascular disorders such as arteriosclerosis, hypercholesterolemia, hypertension, chronic heart failure, in metabolic disorders such as diabetes mellitus, in preeclampsia, erectile dysfunction and other disorders, the biological effects of NO are diminished, thus accelerating progression of these diseases and accompanying vascular lesions. This process can be antagonized by administration of L-arginine.

It has been demonstrated in several clinical and

experimental investigations that the concentration of the endogenous L-arginine analog ADMA in the plasma or serum may rise in the case of the diseases mentioned. Elevated concentrations of ADMA were found in conjunction with peripheral arterial occlusive disease (Böger et al., Circulation 1997; 95: 2068-2074), hypercholesterolemia (Böger et al., Circulation 1998; 98: 1842-1847), hypertension (Surdacki et al., J. Cardiovasc. Pharmacol. 1999; 33: 652-658), chronic renal failure (Kielstein et al., J. Am. Soc. Nephrol. 1999; 10: 594-600) and chronic heart failure. However, it was not possible to infer a cause-effect relationship between elevated ADMA concentrations and these disorders from the results of these studies. ADMA inhibits the formation of NO from L-arginine which is mediated in endothelial cells by the enzyme NO synthase. This would thus explain that the elevated ADMA concentrations might contribute to the progression of the disease process by inhibition of NO formation. Elevated blood glucose concentrations as they occur in diabetes mellitus also reduce the effect of NO, thereby promoting the occurrence of complications of the cardiovascular system. In preeclampsia, a disorder of the NO metabolism leads to constriction of arteries which induces high blood pressure in the mother and poses a risk to the unborn child due to reduced placental perfusion. A deficiency in NO effects moreover constitutes an important cause in erectile dysfunction. In contrast, SDMA which likewise is an endogenous molecule obviously has no inhibitory effect on the activity of NO synthase.

The invention is based on the observation that patients with hypercholesterolemia, peripheral arteriosclerosis, heart failure, chronic renal failure, diabetes mellitus and hypertension have higher ADMA concentrations in the plasma than healthy volunteers. We have now been able

for the first time to demonstrate that ADMA is a factor which proves significant in the prognosis of future progression of diseases: Patients with higher ADMA concentrations have a significantly higher probability to suffer a life-threatening circulatory disturbance or die from it than patients with lower ADMA concentrations (example 1). Patients with high ADMA concentrations are also overall at a significantly higher risk of dying, irrespective of the underlying cause of death (example 2). Moreover, patients with relatively high ADMA concentrations are significantly more frequently diagnosed with thickening of the carotid artery wall than patients with low ADMA concentrations (example 3). Patients with chronic heart failure and elevated ADMA concentrations have a lower maximum oxygen uptake on exertion - an indication of an unfavorable prognosis of future disease progression (example 4).

With ADMA thus presenting a significant factor for the course said diseases take, it is useful to be able to specifically measure the concentration in the individual patient by means of a universally available and rapid diagnostic test and to distinguish it from the SDMA concentration.

Currently available measurement methods for the quantitative determination of ADMA and SDMA in plasma, serum, urine and other biological fluids (tissue extracts, cell culture supernatants, etc.) are all based on the chemical detection method of high pressure liquid chromatography (HPLC). Various modifications of HPLC methods are currently employed for this purpose. However, these methods are very time-consuming and personnel-intensive, costly and therefore not suitable for routine clinical diagnostics.

It is an object of the present invention to detect the probability of the future progression of diseases that are associated with a disorder of the NO metabolism.

This object is achieved according to the invention by detecting a content of endogenous methyl arginines in biological fluids.

In spite of very comprehensive determination of various cardiovascular risk factors known to date, progression of said diseases is detected in a proportion of patients despite the absence of such hitherto known risk factors. The invention has the advantage that at least part of these cases can be explained through the presence of elevated concentrations of the endogenous methyl arginines, thus allowing for an enhanced prognosis of disease progression to be made.

The methods employed so far for detecting ADMA and SDMA are based on the principle of HPLC, which renders them very complex and expensive, thus preventing them from being comprehensively used in clinical routine and remaining reserved for specialized research laboratories. A further object of the invention is therefore to provide a method with the aid of which the endogenous methyl arginines ADMA and SDMA can be quantitatively determined in biological fluids in a simple, inexpensive and clinically universally utilizable manner.

This object is achieved according to the invention in that the content of endogenous methyl arginines in a biological fluid is determined by exposing the latter to antibodies.

Determination of ADMA and SDMA concentrations by means of immunochemical methods (i.e. using monoclonal and

polyclonal antibodies) has, according to the invention, important advantages:

1. Measurement of ADMA and SDMA concentrations using antibody-based methods need not be carried out in highly specialized HPLC laboratories. Immunochemical detection methods such as radio immunoassays, enzyme immunoassays etc. are routinely available in most biochemical laboratories.

2. Measurement with immunochemical detection methods takes less time to perform than measurement by HPLC. The latter method requires complex sample extraction from the respective matrix (plasma, serum, urine or the like) before measurement by HPLC can take place. However, for this parameter to find wide acceptance as disease marker, it is irrefutably necessary for the measured result to be available rapidly when using ADMA or SDMA detection in clinical routine.

3. Immunochemical detection methods may be used not only in clinical routine but also in experimental applications (e.g. immunohisto-chemistry, immunocytochemistry, immunoblotting).

4. According to the present invention, the diagnostic detection of ADMA and SDMA is suitable for obtaining prospective information on a patient's risk of developing a disease or dying ("risk factor").

Monoclonal antibodies are produced according to generally known immunological methods by fusion of immunocompetent cells from sensitized test animals with

myeloma cells and selection of the specific antibody-producing cell clones by means of standard methods. Polyclonal antibodies are produced by obtaining immune serum from immunized test animals according to generally known methods. Production of monoclonal antibodies directed against ADMA and SDMA includes culturing the antibody-producing cell clones, obtaining the conditioned medium comprising the antibodies, and filling and distribution of the antibody solutions. Utilization of antibodies includes their use for diagnostic and scientific purposes, as antibody suspension or as constituent of a diagnostic kit, in the areas of clinical medicine, experimental medicine, veterinary medicine, biology and other biosciences.

The examples below explain the invention:

Example 1

225 patients with chronic renal failure were included in a clinical study. At the beginning of the study, a blood sample was drawn from each patient for determination of ADMA and SDMA and also L-arginine concentrations. The patients were followed up over an average observation period of 26 months (1-35 months). During this time, 57 patients suffered a cardiovascular disease event (myocardial infarction, stroke, peripheral circulatory disturbance or heart-related death). Patients experiencing such a cardiovascular disease event had had significantly higher ADMA concentrations at the start of the study (median 3.2 µmol/l) than patients not developing such an event (median 2.2 µmol/l). In the statistical analysis, ADMA turned out to be an independent predictor of cardiovascular disease events. Where patients were allocated to groups (ADMA < 50th percentile, 51-70th percentile, 71-90th percentile and > 90th percentile)

based on the plasma ADMA concentrations measured at the start of the study, a distinct increase in the frequency of cardiovascular deaths with increasing ADMA concentration was found (figure 1).

Example 2

Amongst 225 patients in whom the ADMA concentration had been determined in a blood sample drawn at the beginning of the study, a total of 57 deaths due to various causes occurred over the course of a follow-up period spanning on average 26 months. Patients who died in the course of the study had significantly higher ADMA concentrations at the beginning (median 3.5 µmol/l) than surviving patients (2.3 µmol/l). ADMA turned out to be a predictor of survival which was independent of other risk factors. Where patients were allocated to groups (ADMA < 50th percentile, 51-70th percentile, 71-90th percentile and > 90th percentile) based on the plasma ADMA concentrations measured at the start of the study, a distinct increase in the overall mortality with increasing ADMA concentration was found (figure 2).

Example 3

For 90 patients, the ADMA concentration was determined in a plasma sample. These patients subsequently underwent high-resolution sonography to measure the thickness of the carotid artery wall. ADMA was an independent influencing factor for intima-media thickness of the carotid artery (figure 3), luminal cross-sectional area and severity of arteriosclerotic lesions in the carotid artery.

Example 4

A blood sample was drawn from 45 patients with chronic heart failure. Measurement of the ADMA concentration yielded an average value of 4.1 ± 0.8 µmol/l, compared with 1.0 ± 0.1 µmol/l in healthy controls. Patients with relatively high ADMA concentrations exhibited reduced NO-mediated vessel dilation ($R = -0.79$) and lower maximum oxygen uptake on exertion ($R = -0.81$; figure 2a). Administration of L-arginine significantly improved NO-mediated vessel dilation; the magnitude of this L-arginine-induced improvement was the higher the higher the ADMA concentration was in the patient concerned ($R = 0.74$; figure 2b).

Patent Claims

1. A method for detecting the probability of the future occurrence or progression of diseases that are associated with a disorder of the NO metabolism, characterized in that a content of endogenous methyl arginines in a biological fluid is detected.

2. The method as claimed in claim 1, characterized in that the disease is coronary heart disease.

3. The method as claimed in claim 1, characterized in that the disease is chronic heart failure.

4. The method as claimed in claim 1, characterized in that the disease is erectile dysfunction.

5. The method as claimed in claim 1, characterized in that the disease is a peripheral arterial circulatory disturbance.

6. The method as claimed in claim 1, characterized in that the disease is chronic renal failure.

7. The method as claimed in claim 1, characterized in that the disease is a cerebral ischaemic disease.

8. The method as claimed in claim 1, characterized in that the disease is diabetes mellitus.

9. The method as claimed in claim 1, characterized in that the disease is preeclampsia.

10. The method as claimed in one of claims 1 to 9, characterized in that the level of an ADMA concentration in the biological fluid is

determined.

11. The method as claimed in one of claims 1 to 10, characterized in that an L-arginine to ADMA concentrations ratio in the biological fluid is determined.

12. The method as claimed in one of claims 1 to 10, characterized in that the level of an SDMA concentration in the biological fluid is determined.

13. The method as claimed in one of claims 1 to 12, characterized in that an L-arginine to SDMA concentrations ratio in the biological fluid is determined.

14. The method as claimed in one of claims 1 to 13, characterized in that an ADMA to SDMA concentrations ratio in the biological fluid is determined.

15. The method as claimed in one of claims 1 to 14, characterized in that the content of endogenous methyl arginines in a biological fluid is determined by exposing it to antibodies.

16. The method as claimed in claim 15, characterized in that the biological fluid comprising the endogenous methyl arginine is exposed to monoclonal antibodies.

17. The method as claimed in claim 15 or 16, characterized in that the biological fluid comprising the endogenous methyl arginine is exposed to polyclonal antibodies.

18. The method as claimed in claim 17, characterized in that the monoclonal antibodies are obtained by culturing the antibody-producing cell clones and obtaining a solution comprising the antibodies.

19. The method as claimed in claim 17, characterized in that the polyclonal antibodies are generated by obtaining immune serum from immunized test animals.

20. An agent for carrying out methods 1 to 19, characterized in that the biological fluid consists of plasma.

21. An agent for carrying out methods 1 to 19, characterized in that the biological fluid consists of serum.

22. An agent for carrying out methods 1 to 19, characterized in that the biological fluid consists of urine.

23. An agent for carrying out methods 1 to 19, characterized in that the biological fluid consists of tissue extracts.

24. An agent for carrying out methods 1 to 19, characterized in that the biological fluid consists of histological preparations.

25. An agent for carrying out methods 1 to 19, characterized in that the biological fluid consists of cytological preparations.

Figure 1

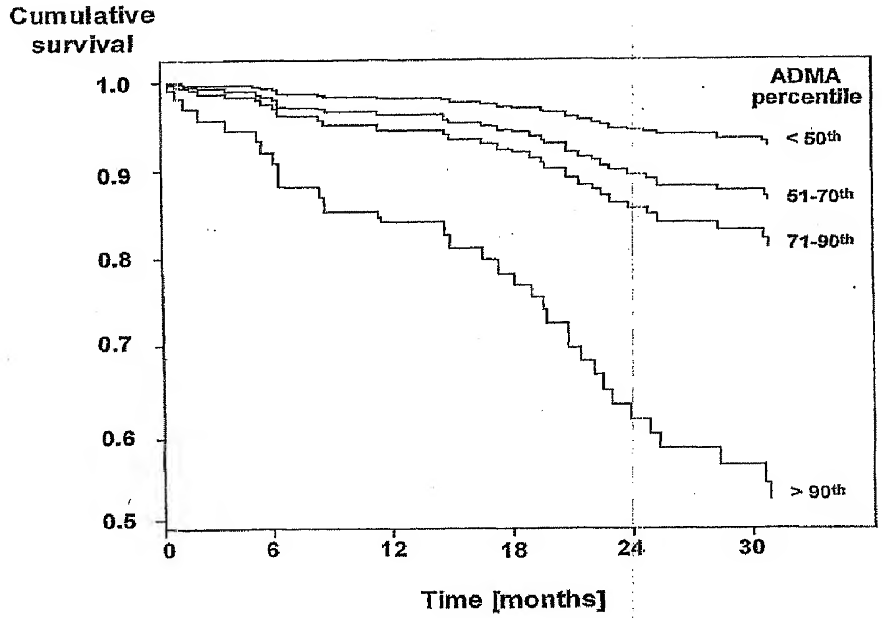
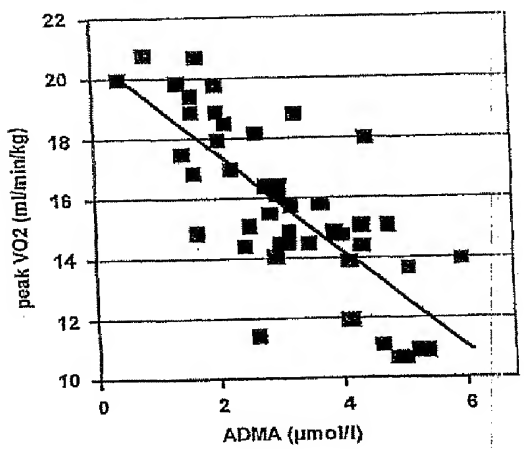
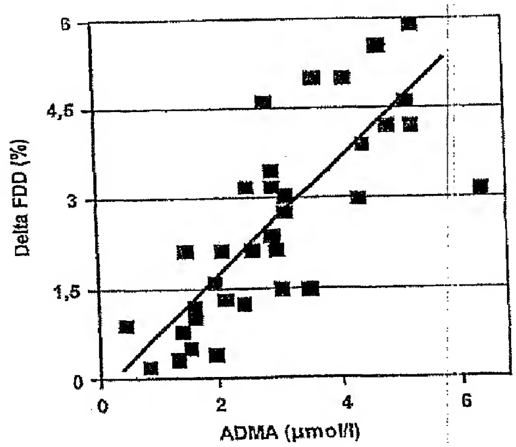

Cumulative survival
1.0
0.9
0.8
0.7
0.6
0.5
0
6
12
18
24
30
Time [months]
ADMA percentile
< 50th
51-70th
71-90th
> 90th

Figure 2

a.


22
20
18
16
14
12
10
peak VO2 (ml/min/kg)
0
2
4
6
ADMA (µmol/l)

b.


6
4,5
3
1,5
0
Delta FDD (%)
0
2
4
6
ADMA (µmol/l)

[Corrector: please append figures and edit as follows:

Abbildung 1	→	**Figure 1**
Abbildung 2	→	**Figure 2**